Electronic Supplementary Material (ESI) for Environmental Science: Advances.

SUPPORTING INFORMATION

Wastewater-based surveillance of COVID-19 and removal of SARS-CoV-2 RNA across a major wastewater treatment plant in San Antonio, Texas

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RT-ddPCR Detailed Procedure

Reverse transcription droplet digital PCR (RT-ddPCR) was used to measure SARS-CoV-2 RNA copies using the U.S. Centers for Disease Control and Prevention (CDC) N1 and N2 primer/probe sets. The sets of primers and probe (2019-nCoV RUO Kit) were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa). The RNA extracts were thawed on ice, then quickly used for RT-ddPCR assays. The RT-ddPCR experiments were conducted on a Bio-Rad QX200™ Droplet Digital™ PCR System using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad). For each ddPCR well, a final reaction volume of 20 µL was used that comprised 5 µL 1 × One-Step RT-ddPCR Supermix (Bio-Rad), 2 µL reverse transcriptase (Bio-Rad), 1 µL 300 mM DTT, 2 µL primers/probe mix (final concentrations were 670 and 170 nM primers and probe, respectively), 5 µL RNase-free water, and 5 µL RNA template. For each assay, duplicate no-template controls (NTC) were used to check for cross contamination. To test for PCR inhibition, selected RNA extracts were run at both undiluted and 10-fold dilution for both N1 and N2 assays, and confirmed that there was no PCR inhibition. PCR inhibition tests were done on the first three set of samples for each treatment stage. PCR inhibition tests resulted in a Ct value proportional to a 10-fold dilution relative to the undiluted RNA templates, suggesting that PCR inhibition did not interfere with the amplification efficiency. For droplet generation, 20 µL of reaction mixture and 70 µL of QX200™ Droplet Generation Oil for Probes was transferred to DG8 gaskets and run on QX200 Droplet Generator (Bio-Rad). The resulting oil emulsion was then carefully pipetted to a new ddPCR 96-well plate and PX1 PCR Plate Sealer (Bio-Rad) was then used to foil-seal the plate. ddPCR plate was transferred to the C1000 Touch™ thermocycler (Bio-Rad) and run using following conditions: 60 min at 50°C for reverse transcription, 10 min at 95°C for enzyme activation, 40 cycles each of 30 s at 94°C for denaturation and 1 min at 55°C for

annealing/extension (ramp rate of ~2°C/s), finally 10 min at 98°C for enzyme deactivation (Gonzalez et al. 2020). For droplet stabilization purposes, a 15-min 4°C cool down step was added following the end of thermal cycling. Droplets were read using a QX200 Droplet Reader (Bio-Rad).

For the N1 and N2 assays, the limit of detection (LOD) was defined as the lowest concentration likely to be reliably distinguished from the NTC. If the NTC was zero (i.e., no positive droplet in any of the replicates), then the LOD was set at 3 positive droplets as a conservative threshold. Limits of quantification (LOQ) were calculated by running serial dilutions of the 2019-nCoV_N_Positive Control in triplicates distributed over multiple days, and determined as the lowest concentration at which the relative standard deviation was ≤30% between triplicate assay measures and the difference between the calculated and expected concentrations was ≤30%. For both N1 and N2 assays, the LOD was set at 3 positive droplets and the LOQ was 10 positive droplets.

RNA recovery efficiency

To determine virus recovery efficiency of our concentration and extraction methodology, an attenuated vaccine strain of bovine coronavirus (Calf-Guard, Zoetis, Parsippany, NJ) was used as a surrogate viral RNA target. Wastewater influent samples were spiked with 10^6 copies of bovine coronavirus (BCoV) per liter of wastewater, directly before sample acidification and/or filtration. RNA extraction was performed using the RNeasy Power Microbiome Kit (Qiagen) and RT-ddPCR quantification assay was performed on the bovine coronavirus target gene. The 20 µL reaction volume was constituted from 5 µL 1 × one-step RT-ddPCR Supermix (Bio-Rad), 2 µL reverse transcriptase (Bio-Rad), 1 µL 300 mM DTT, 1.2 µL forward and reverse primers and 0.4 µL probe

(with final concentrations of 600 and 200 nM, respectively), 4.2 µL RNase-free water, and 5 µL RNA. No template control (NTC) and positive control were included in each run. Quantification of bovine coronavirus was performed in a similar manner to the SARS-CoV-2 targeted assay, with respective reaction mixture constituents and thermal cycling conditions. The resulting recovery efficiency was calculated using the ratio of bovine coronavirus concentration recovered by concentration seeded. Recovery efficiency was quantified using all the samples and processed in duplicate. Random samples (n = 3) without BCoV added were tested to confirm there is no natural BCoV in the wastewater samples.

Table S1. SARS-CoV-2 Wastewater Concentration Calculation (copies per liter wastewater)

Equation 1	$$N1\left(\frac{copy\ number}{liter}\right) = \frac{C}{V_{RNA}} \times D \times \frac{V_{RNA\ eluted}}{V_{wastewater}}$$ *C*, RNA copy number per ddPCR well V_{RNA}, volume of RNA added to reaction *D*, dilution factor $V_{RNA\ eluted}$, volume of RNA eluted during extraction $V_{wastewater}$, volume of wastewater filtered in liters
Equation 2	$$N2\left(\frac{copy\ number}{liter}\right) = \frac{C}{V_{RNA}} \times D \times \frac{V_{RNA\ eluted}}{V_{wastewater}}$$ *C*, RNA copy number per ddPCR well V_{RNA}, volume of RNA added to reaction *D*, dilution factor $V_{RNA\ eluted}$, volume of RNA eluted during extraction $V_{wastewater}$, volume of wastewater filtered in liters

Table S2. RT-ddPCR raw data for SARS-CoV-2 N1 and N2 assays tested with raw influent samples.

Sample Date	Sample ID	N1 (copies/uL)	N1 Total Droplets	N1 Positive Droplets	N2 (copies/uL)	N2 Total Droplets	N2 Positive Droplets
4/12/2021	R1	1.75	20830	31	0.90	26269	20
4/14/2021	R2	12.16	23821	245	14.41	25142	306
4/19/2021	R3	1.84	25635	40	1.87	19555	31
4/21/2021	R4	1.03	18226	16	0.89	25120	19
4/26/2021	R5	2.04	21924	38	1.45	23464	29
4/28/2021	R6	3.66	22563	70	2.64	20508	46
5/3/2021	R7	4.39	23336	87	3.47	23115	68
5/5/2021	R8	1.75	24943	37	1.68	26578	38
5/10/2021	R9	2.20	30497	57	2.32	31981	63
5/12/2021	R10	0.87	27157	20	1.17	27186	27
5/17/2021	R11	2.98	26128	66	3.06	28090	73
5/19/2021	R12	0.79	22223	15	0.32	29444	8
5/24/2021	R13	0.23	30074	6	0.15	30471	4
5/26/2021	R14	3.20	29776	81	3.54	27304	82
5/31/2021	R15	0.84	21039	15	0.80	14787	10
6/2/2021	R16	0.44	18734	7	0.21	11173	2
6/7/2021	R17	3.23	14945	41	1.08	10896	10
6/9/2021	R18	1.01	26811	23	0.13	27592	3
6/14/2021	R19	2.44	25623	53	1.89	26192	42
6/16/2021	R20	0.87	23035	17	0.00	30094	0
6/21/2021	R21	2.19	26915	50	0.26	27078	6
6/23/2021	R22	1.14	25918	25	0.31	26492	7
6/28/2021	R23	2.13	26518	48	1.34	23776	27
6/30/2021	R24	2.22	20712	39	1.17	18072	18
7/5/2021	R25	0.32	25718	7	0.05	21844	1
7/7/2021	R26	0.45	26103	10	0.70	23477	14
7/12/2021	R27	7.29	28976	179	4.43	24499	92
7/14/2021	R28	13.77	27854	324	9.97	29512	249

Table S2 Continued. RT-ddPCR raw data for SARS-CoV-2 N1 and N2 assays tested with raw influent samples.

Sample Date	Sample ID	N1 (copies/uL)	N1 Total Droplets	N1 Positive Droplets	N2 (copies/uL)	N2 Total Droplets	N2 Positive Droplets
7/19/2021	R29	2.62	26924	60	1.94	23718	39
7/21/2021	R30	6.49	26346	145	1.92	22054	36
7/26/2021	R31	6.98	22832	135	2.95	23993	60
7/28/2021	R32	15.58	33362	439	2.82	28447	68
8/2/2021	R33	27.94	27692	650	5.19	29523	130
8/4/2021	R34	27.19	34529	789	5.47	31917	148
8/9/2021	R35	15.08	16961	216	6.2	24561	129
8/11/2021	R36	8.38	24938	177	3.14	32979	88
8/16/2021	R37	11.55	22933	224	3.41	25575	74
8/18/2021	R38	12.26	24410	253	3.35	29887	85
8/23/2021	R39	22.59	24974	475	9.76	29522	244
8/25/2021	R40	3.29	30476	85	2	30653	52
8/30/2021	R41	8.54	23215	168	6.88	29854	174
9/1/2021	R42	11.24	23565	224	9	29528	225
9/6/2021	R43	2.53	29783	64	3.43	26102	76
9/8/2021	R44	5.14	34885	152	1.66	31279	44
9/13/2021	R45	8.61	34408	251	4.03	28971	99
9/15/2021	R46	1.63	34602	48	0.54	24172	11
9/20/2021	R47	2.32	26344	52	0.61	28743	15
9/22/2021	R48	5.15	27247	119	3.96	25603	86
9/27/2021	R49	2.51	26737	57	2.09	27090	48
9/29/2021	R50	9.32	25209	199	8.77	23826	177
10/4/2021	R51	6.78	18101	104	4.54	24687	95
10/6/2021	R52	9.98	22492	190	3.4	29762	86
10/11/2021	R53	4.42	19981	75	3.09	24814	65

Table S2. Continued. RT-ddPCR raw data for SARS-CoV-2 N1 and N2 assays tested with raw influent samples.

Sample Date	Sample ID	N1 (copies/uL)	N1 Total Droplets	N1 Positive Droplets	N2 (copies/uL)	N2 Total Droplets	N2 Positive Droplets
10/13/2021	R54	0.5	25754	11	0.19	31074	5
10/18/2021	R55	1.78	27076	41	0.59	30084	15
10/20/2021	R56	2.76	25170	59	0.91	24569	19
10/25/2021	R57	1.56	23440	31	0.51	25605	11
10/27/2021	R58	0.11	21846	2	0.19	24808	4
11/1/2021	R59	0.76	26400	17	0.29	24633	6
11/3/2021	R60	1.3	23511	26	0.19	24556	4
11/8/2021	R61	5.15	24950	109	4.12	22004	77
11/10/2021	R62	3.22	21564	59	1.27	24041	26
11/15/2021	R63	0.67	22998	13	0	25100	0
11/17/2021	R64	3.91	27726	92	0.1	23246	2
11/22/2021	R65	6.93	27937	164	2.9	23182	57
11/24/2021	R66	5.25	28070	125	1.24	29416	31
11/29/2021	R67	0.91	24477	19	0.3	31378	8

Table S3. SARS-CoV-2 N1 and N2 concentrations (copies/L) detected in Raw Influent (R), Primary Effluent (PE), Secondary Effluent (SE) and Final Effluent (F) samples over a period of four months.

Date	SARS-CoV-2 N1 concentration (copies/L)				SARS-CoV-2 N2 concentration (copies/L)			
	Raw Influent	Primary Effluent	Secondary Effluent	Final Effluent	Raw Influent	Primary Effluent	Secondary Effluent	Final Effluent
4/12/2021	1752	937	BDL[a]	BDL	896	697	BDL	0
4/14/2021	12163	2802	0	0	14407	3595	BDL	0
4/19/2021	1837	1658	0	BDL	1867	1075	0	0
4/21/2021	1033	848	0	0	890	503	BDL	0
4/26/2021	2041	204	0	0	1455	393	0	0
4/28/2021	3656	1253	0	0	2642	1788	BDL	0
5/3/2021	4394	867	BDL	0	3466	1157	0	BDL
5/5/2021	1746	1998	0	0	1683	1430	0	0
5/10/2021	2201	533	0	0	2320	739	0	0
5/12/2021	867	194	BDL	0	1169	533	BDL	BDL
5/17/2021	2976	1116	BDL	0	3061	1016	BDL	0
5/19/2021	794	141	BDL	0	320	BDL	BDL	BDL
5/24/2021	235	BDL	BDL	BDL	154	0	BDL	0
5/26/2021	3205	556	BDL	BDL	3539	518	BDL	0
5/31/2021	839	493	BDL	BDL	796	383	BDL	0
6/2/2021	440	1092	0	0	211	580	BDL	BDL
6/7/2021	3232	149	0	0	1080	106	BDL	BDL
6/9/2021	1010	982	BDL	BDL	128	403	0	0
6/14/2021	2436	523	BDL	0	1888	429	BDL	0
6/16/2021	869	283	0	0	0	0	0	0
6/21/2021	2188	353	0	0	261	BDL	BDL	0
6/23/2021	1135	295	0	0	311	BDL	BDL	0
6/28/2021	2131	BDL	480	BDL	1337	505	0	0
6/30/2021	2217	894	201	0	1172	290	BDL	0
7/5/2021	320	668	BDL	BDL	BDL	532	0	0
7/7/2021	451	2272	1541	BDL	702	1452	BDL	0
7/12/2021	7290	6415	180	0	4426	4368	0	0
7/14/2021	13765	1239	422	0	9968	465	BDL	0
7/19/2021	2624.	1223	210	0	1936	1968	BDL	0
7/21/2021	6492	4926	BDL	0	1921	1500	BDL	0
7/26/2021	6976	6664	374	BDL	2945	1950	BDL	0

[a] BDL: Below detection limit.

Table S4. Examples of RT-ddPCR positive and negative results.

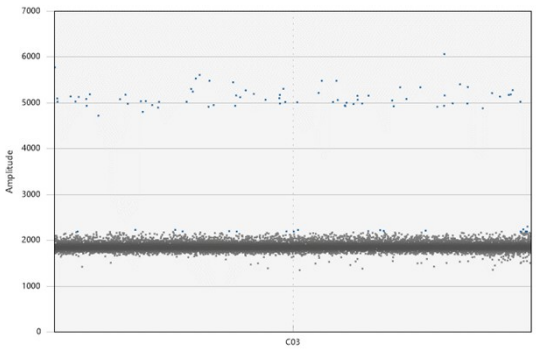
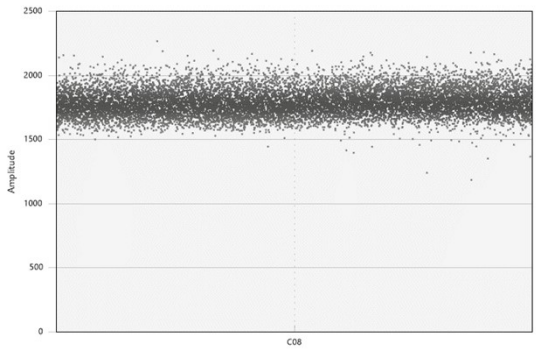
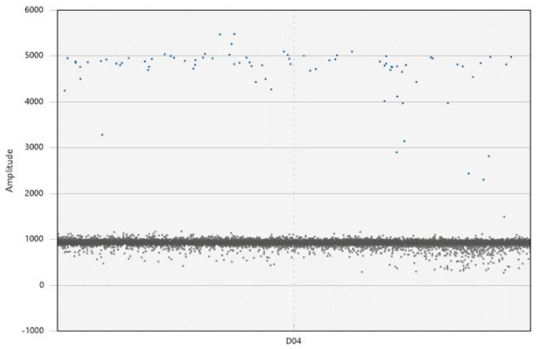
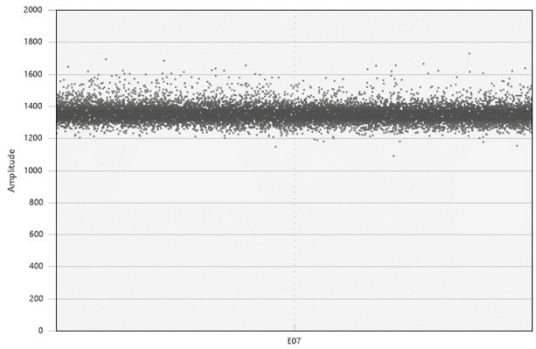
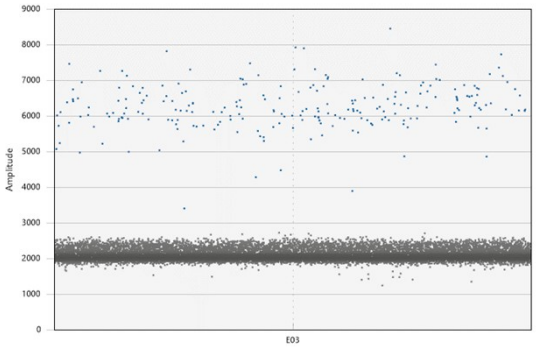
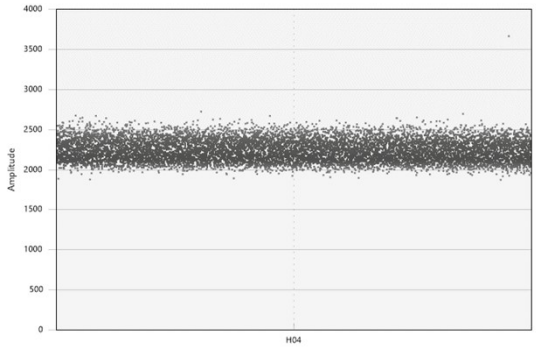
Assay	Positive reaction	Negative Reaction
N1		
N2		
BCoV		

Table S5. Digital MIQE Checklist.

ITEM TO CHECK	PROVIDED	COMMENT
	Y/N	
1. SPECIMEN		
Detailed description of specimen type and numbers	Y	Methods "Description of the wastewater treatment plant and sample collection"
Sampling procedure (including time to storage)	Y	Methods "Description of the wastewater treatment plant and sample collection"
Sample aliquotation, storage conditions and duration	Y	Methods "Description of the wastewater treatment plant and sample collection"
2. NUCLEIC ACID EXTRACTION		
Description of extraction method including amount of	Y	Methods "Virus concentration and nucleic acid extraction"
Volume of solvent used to elute/resuspend extract	Y	Methods "Virus concentration and nucleic acid extraction"
Number of extraction replicates	Y	Methods "Virus concentration and nucleic acid extraction"
Extraction blanks included?	Y	Methods "Virus concentration and nucleic acid extraction"
3. NUCLEIC ACID ASSESSMENT AND STORAGE		
Method to evaluate quality of nucleic acids	Y	Methods "Virus concentration and nucleic acid extraction"
Method to evaluate quantity of nucleic acids (including molecular weight and calculations when using mass)	N	Not performed
Storage conditions: temperature, concentration, duration, buffer, aliquots	Y	Methods "Virus concentration and nucleic acid extraction"
Clear description of dilution steps used to prepare working DNA solution	Y	Methods "RT-ddPCR"
4. NUCLEIC ACID MODIFICATION		
Template modification (digestion, sonication, pre-	N	Not performed
Details of repurification following modification if performed	N	Not performed
5. REVERSE TRANSCRIPTION		
cDNA priming method and concentration	N	Not applicable
One or two step protocol (include reaction details for two	Y	One step; Methods "RT-ddPCR"
Amount of RNA added per reaction	Y	Methods "RT-ddPCR"; Supporting Information
Detailed reaction components and conditions	Y	Methods "RT-ddPCR"; Supporting Information
Estimated copies measured with and without addition of RT*	N	Not performed
Manufacturer of reagents used with catalogue and lot	Y	Methods "RT-ddPCR"; Supporting Information; catalogue no. not provided
Storage of cDNA: temperature, concentration, duration,	N	Not applicable
6. dPCR OLIGONUCLEOTIDES DESIGN AND TARGET INFORMATION		
Sequence accession number or official gene symbol	N	Reference provided
Method (software) used for design and *in silico* verification	N	Reference provided
Location of amplicon	N	Reference provided
Amplicon length	N	Reference provided
Primer and probe sequences (or amplicon context sequence)**	Y	Table 1
Location and identity of any modifications	Y	Table 1
Manufacturer of oligonucleotides	Y	Methods "RT-ddPCR"; Supporting Information
7. dPCR PROTOCOL		
Manufacturer of dPCR instrument and instrument model	Y	Methods "RT-ddPCR"; Supporting Information
Buffer/kit manufacturer with catalogue and lot number	Y	Methods "RT-ddPCR"; Supporting Information
Primer and probe concentration	Y	Methods "RT-ddPCR"; Supporting Information
Pre-reaction volume and composition (incl. amount of	Y	Methods "SARS-CoV-2 viral RNA quantification"
Template treatment (initial heating or chemical denaturation)	N	Not performed
Polymerase identity and concentration, Mg++ and dNTP concentrations***	Y	Methods "RT-ddPCR"; Supporting Information
Complete thermocycling parameters	Y	Methods "RT-ddPCR"; Supporting Information
8. ASSAY VALIDATION		
Details of optimisation performed	N	Not performed
Analytical specificity (vs. related sequences) and limit of blank (LOB)	N	Not performed
Analytical sensitivity/LoD and how this was evaluated	Y	Methods "RT-ddPCR"; Supporting Information
Testing for inhibitors (from biological matrix/extraction)	Y	Methods "RT-ddPCR"; Supporting Information
9. DATA ANALYSIS		
Description of dPCR experimental design	Y	Methods "RT-ddPCR"; Supporting Information
Comprehensive details negative and positive of controls (whe	Y	Methods "RT-ddPCR"; Supporting Information
Partition classification method (thresholding)	Y	Methods "RT-ddPCR"; Supporting Information
Examples of positive and negative experimental results	Y	Table S4
Description of technical replication	Y	Methods "RT-ddPCR"; Supporting Information
Repeatability (intra-experiment variation)	Y	Methods "RT-ddPCR"; Supporting Information
Reproducibility (inter-experiment/user/lab etc. variation)	N	Not performed
Number of partitions measured (average and standard deviation)	N	Not performed
Partition volume	N	Not performed
Copies per partition (λ or equivalent) (average and standard deviation)	N	Not performed
dPCR analysis program (source, version)	Y	Methods "RT-ddPCR"; Supporting Information
Description of normalisation method	N	Not performed
Statistical methods used for analysis	Y	Methods "RT-ddPCR"; Supporting Information; "Statistical analyses"
Data transparency	upplemental files:	Table S2 and S3

Table S6. Results for PCR inhibition tests.

Sample	SARS-CoV-2 N1 RNA conc. (copies/L)		SARS-CoV-2 N2 RNA conc. (copies/L)	
	Undiluted	**10-fold diluted**	**Undiluted**	**10-fold diluted**
Raw influent	1752	173	896	79
Raw influent	12163	1312	14407	1522
Raw influent	1837	172	1867	121
Primary effluent	937	80	697	65
Primary effluent	2802	219	3595	361
Primary effluent	1658	161	1075	119
Secondary effluent	BDL	0	BDL	0
Secondary effluent	0	0	BDL	0
Secondary effluent	0	0	0	0
Final effluent	BDL	0	0	0
Final effluent	0	0	0	0
Final effluent	BDL	0	0	0